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Histochemical Demonstration of Glycogen Synthesis in vitro.

By

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Introduction

Although the knowlege of the histochemistry of enzymes has advanced remarkably in recent years, there are still some difficult problems in this scientific field. As an example of these problems we can point to a histochemical study of an enzyme which acts on substances of high molecular weight such as protein and glycogen. Such studies have been started by the present authors and some of the results have been reported alredy. Concerning these studies, we attempted prove glycogen synthesis in tissue sections by using the histochemical method. Yin and Sun (1947) detected starch formation on starch-free, water-saoked sections of soybean as an indicator of phosphorylase. In recent years some investsgators, Takeuchi and Kuriaki (1954) and Goldberg, Wade and Jones (1952), also have done some experiments with same idea to dimonstrate phosphorylase in animal tissues. Our experiments were performed independently of theirs and have already been reported in other papers. All these experiments resembled each other. But our experimental purpose did not concern phosphorylation but the synthesis of polysaccaride of high molecular weight. The experimental methods of each investsgator are different. Moreover, as an important result we proved glycogen synthesis by using fructose-monophosphate as a raw material. This finding was not noted by other investigators. Therfore we will describe our own experiments and their results in this reports.

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Histochemical Experiments on Glycogen Synthesis from fructose-1-monophosphate using tissue blocks in vitro.

We used rat tissues for this experiment. These rats were starved for 12 to 24 hours. Too much starvation was not suitable. After sacrificing these animals tissues were removed. Then the tissues were immediately cut by hand using a razor blade. The desirable thickness of tissues blocks is 1 to 2 mm. These tissue blocks were incubated immediately in the following substrate mixture at 37°C for four hours.

After incubation, the blocks were fixed in Carnoy's solution, then washed and dehydrated with alcohol in the usual manner and embeded in paraffin. Glycogen was stained with Best's Carmine solution, and periodic acid Schiff (PAS). Feulgen-Bauer method was also tried and was combined with the digestion test with saliva.

The substrate mixture giving best results is; 250 mg of fructore-1-monophosphate (Ca salts) dissolved in 22.5 cc of distilled water and added to 22.5 cc of a saturated water solution of sodium acetate. This mixture is then corrected to pH 6.5 and 7.3 with N/10 HCl solution.

Experimental Results

After starvation for 12 to 24 hours, carmine stainable glycogen disappeared from the liver of these animals (Fig. 1). PAS staining of the same tissue sections showed a number of glycogenposisive cells still present. Some of these cells contained a large amount of PAS positive substances, namely polysaccharide, while other cells contained only a slight amount in their protoplasm (Fig. 2). The tissue sections of the liver from the same individuals following incubation in the substrate mixture stained intensly with Best' Carmine solution (Fig. 3, 4), and also with PAS stained. Carmine stainable substances were contained in the protoplasm of liver cells in various amounts. The most interesting feature is the crowding of this substances around the nuclei (Fig. 4). This peculiar staining was also proved by PAS staining.

Carmine stainable substances of the incubated tissue sections lost their stainability after digestion with saliva. Polysaccharide synthesis is also noted in the epithelium of the outer layer of the intestinal mucosa.

Additional Experiments and Results.

Experiment which used glucose or fructose with inorganic phosphate as a substrate intead of fructose-1-monophosphate in the mixture solution showed no glycogen synthesis. In the other experiments, in which a small amount of adenosinetriphosphate was added to these solutions glycogen synthesis could not be detected. Glucose-1-monophosphate is acceptable as a substrate resulting in similar

synthesis but, in our experiment, this is somewhat inferior and fails to show the characteristic figure around the nuclei of liver cells.

Some of the conditions of the substrate mixture were also investigated. On the kinds of minerals and their concentration we reached no conclusion. The hydrogen ion concentration of the substrate mixture may vary considerably as above mentioned. The influence of insulin on our experiments was not apparent.

Stainability of Glycogen.

We devised a series of experiments to study the stainability of glycogen. Glycogen war dissolved in phosphate buffer solution, then saliva was added to this solution, and incubated at 37°C for a few minutes to two hours. After a definite incubations time a small amount of this solution was sucked up from a glass vessel and put in to a test tube. Then alcohol was added untill the precipitation was complete. The precipitate was washed with alcohol and dried at room temperature. The precipitate was mixed with liquid protein (white of egg) and smeared on a slide, dried, and fixed in Carnoy's solution. These precipitates were stained with Best's Carmine, periodic acid Schiff, and Feulgen-Bauer's methods.

Best's Carmine stained the original glycogen and the precipitates of short time incubations deeply but degree of intensity diminished proportionately to the incubation times. On the other hand, in the periodic acid Schiff reaction the intensity of the colours of precipitates are almost independent of incubation times. Feulgen-Bauer's technique results in a similar figure to that of periodic acid Schiff reaction but not so clear.

From this expriment we can conclude that that Best's Carmine will stain only glycogen or polysaccharides of high molecular weight, but periodic acid Schiff technique demonstrates glycogen and polysaccharides of high and also low molecular weights.

Discussion

Best's Carmine solution, as mentioned above, will stain glycogen or polysaccharides of high molecular weight but not of low molecular weight. On the contrary, periodic acid Schiff stained glycogen and also other saccharides of relatively low molecular weight. In our experiment, the glycogen of liver cells of rats, which is stainable by Best's Carmine solution disappeared following starvation of 12 to 24 hours. But other saccharides remain which are stained by periodic acid Schiff method. It seems to be certain that polysaccharides of high molecular weight disappear during starvation of such a degree but not saccharides of low molecular weight. After incubation of such liver slices, Carmine stainable substances will appear again. This phenomena must be due to synthesis of glycogen in vitro.

As is well known, glycogen can be synthesized by polymerisation of glucose-monophosphate. In our experiments, synthesis of glycogen or polysaccharides of high molecular weight from fructose-monophosphate was proved. Although the

mechanism of glycogen synthesis is not clear, polymerization seems to occur by phosphrylation of sugar phosphate. It is conjectured that this comes about by the interconversion of raw materials. The interconversion of glucose- and fructose-furanose-6-monophosphate is catalysed by oxoisomerase and the interconversion of glucose-6-monophosphate and glucose-1-phosphate by phosphoglucomutase. Therefore some sugar phosphates can be raw metersals of glycogen in vivo and also in vitro. Non-phosphated monoscacharide, even with adenosine triphosphate, in our experiments, failed to be a raw material. The conditions of this experiments war probably unsuitable for synthesis. It in said that the depolymerization of glycogen or polysaccharide to monosaccharide is by phosphorlysis and this biochemical mechanism is catalysed by an enzyme phosphorylase. Phosphorylase activities are said to be reversible. Yin and Sun have attempted to demonstrate of phosphorylase by plant tissue. Takeuchi and Kuriaki have recently attempted with same thing with animal tissues. Goldberg, Wade and Jones described a histochemical method for determing phosphosphorylase with frozen tissue sections. Their idea was the same as ours but their purpose was to demonstrate phosphorylase. We described our method in 1954 independently of Goldberg and others. We started this study from the stand point of enzymatic histochemistry on substances of high molecular weight. Our experiments are similar to Goldbergs but not identical. In our experiments, the synthesis war performed by tissue blocks but not by frozen sections. Goldberg tested only glucose-1-phosphate as a raw material. We, as well as these other investigators, noticed the synthesis of polysacchride in the sections as a final product under the microscope but not phosphate transmission. So it seems to be not reasonable to use the name of phosphorylase in such experiments. We call our experiment, therefore, simply glycogen synthesis. Marsh and Miller (1953) war demonstrated a glycogen synthesis by rat kidney slices in vitro and detected by biochemical method. Their experiments indicate the synthesis were succesful by use of glucose. It seems to be no experiment of histochemistry of glycogen synthesis by use of fructose monophosphate except of ours.

Summary

Histochemical demonstration of glycogen synthesis was attempted. In these experiments, tissue blocks were incubated in a substrate solution. Fresh tissues were removed from starved rats. These tissues contained almost no stainable substances by Best's Carmine solution but after incubation the tissues of the same individuals showed many substances. Best's Carmine will stain only polysaccharides of high molecular weight but not those of lower molecular weight. On the other hand, periodic acid Schiff method reacts with polysaccharide of both high and low molecular weights. We proved the synthesis of polysaccharide of high molecular weight from fructose menophosphate.

Explanation of the plates.

Fig. 1. Rat liver after 12 hours starvation. Stained with Best's Carmine Solution. Counterstained with hematoxylin. There is no carmine stainable substance.

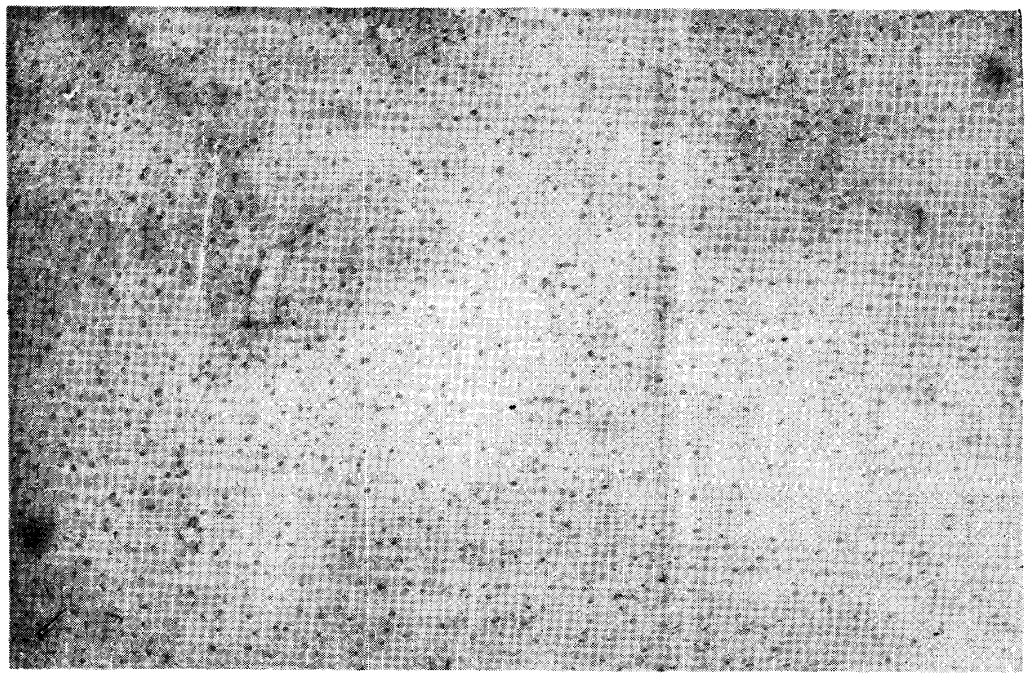


Fig. 2. Same section as Fig. 1. Stained with periodic acid Schiff method. There are scattered positive liver cells of various degrees of density.

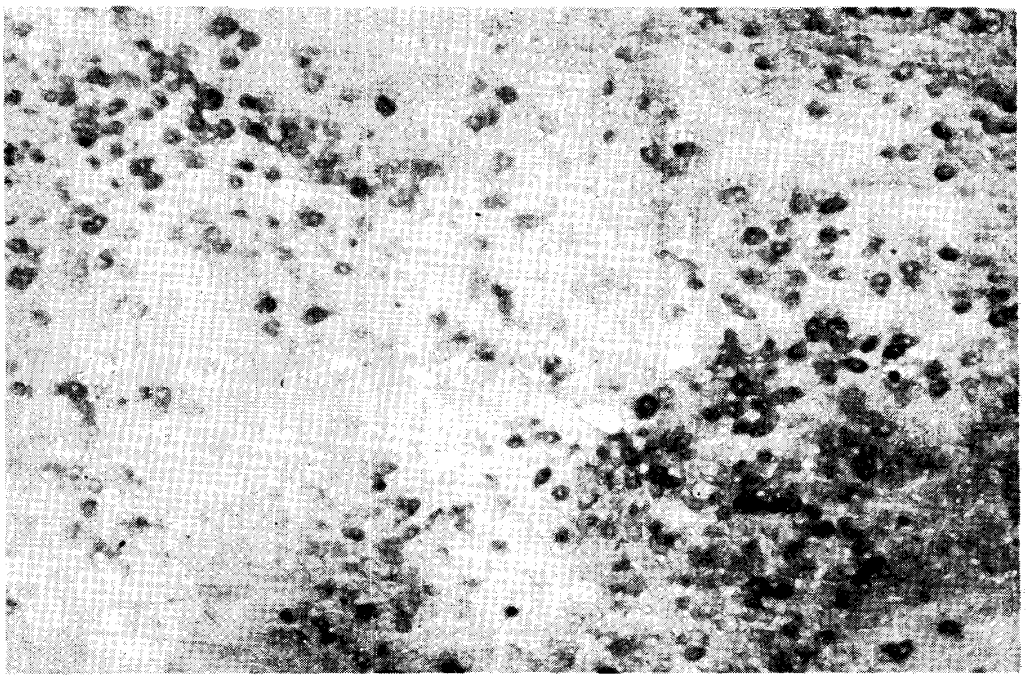


Fig. 3. Rat liver from the same individuals as Fig. 1. but prepared after incubation. Stained with Best's Carmine solution. Stainable substances are deposited distinctly.

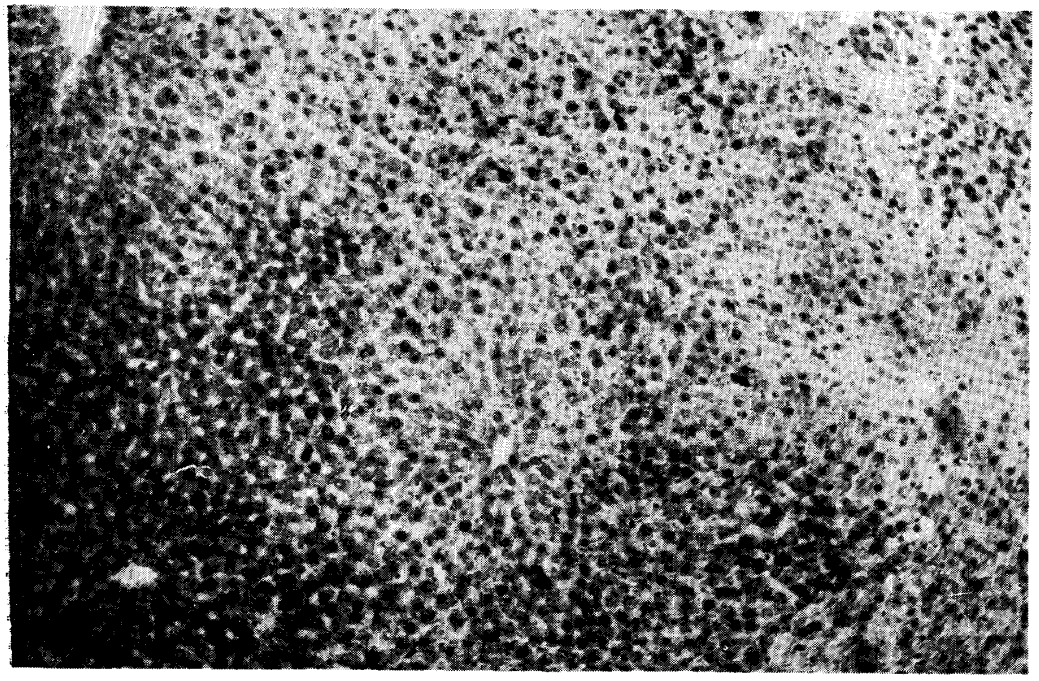
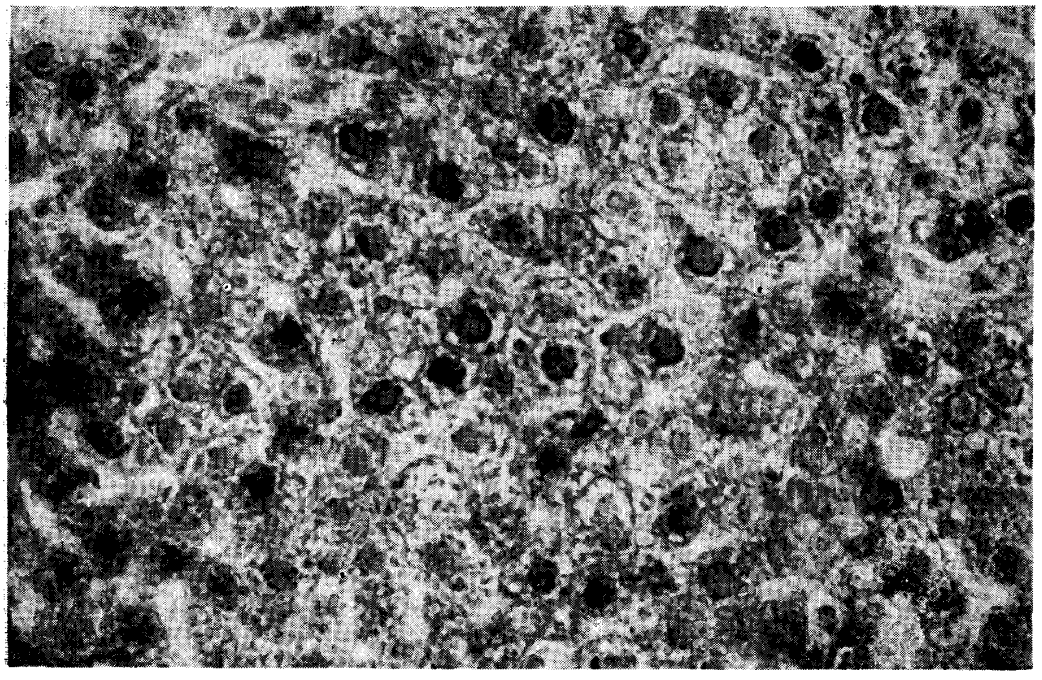


Fig. 4. Large magnification of the same slice as Fig. 3. Carmine stainable substances, glycogen of high molecular weight, are scattered in the protoplasm of the liver cells in various degrees of density, especialy concentrated around the nuclei.



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